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A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents

I. The Stability and Lability of Spontaneous Frequency

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Summary. 1. The circadian pacemakers controlling activity rhythms in four species of rodents are compared, as freerunning systems in constant darkness. In analyzing their stability the distinction is made between (1) spontaneous day-to-day instability of frequency, and (2) a longer-term lability, some of which is traceable to identified causes.

2. Serial correlation analysis indicates that the precision (day-to-day stability) of the pacemaker's period is ca. twice as good (estimated s.d. = 0.6% of $\bar{\tau}$ in *Mus musculus*) as the already remarkable precision of the activity rhythm it drives (average s.d. = 1.2% of $\bar{\tau}$).

3. Identifiable causes of long-term lability include age and several features of prior entrainment by light. The period and photoperiod of a light cycle have a predictable influence on the subsequent freerunning period (τ) of the pacemaker; they cause "after-effects". So do single light pulses causing a phase-shift in the freerunning system. Constant light also has an after-effect opposite in sign from the after-effect of long photoperiods.

4. After-effects of "skeleton" photoperiods support the hypothesis that the transitions of light to darkness vv. are involved in the entrainment process which leads to changes in τ .

5. Both day-to-day instability and long term lability are most pronounced in species (*Peromyscus maniculatus*, *Mus musculus*) whose $\bar{\tau}$ is considerably shorter than 24 h; they are least pronounced in hamsters whose $\bar{\tau}$ is indistinguishably close to 24 h.

6. The differences between the species in τ and its lability are paralleled by differences in pacemaker lability as measured in light-induced after-effects and in the extent of changes with age. The species evidently differ in the "tightness" with which τ is homeostatically conserved.

I. Introduction

The work reported in this and four subsequent papers was designed to ask whether the general model of non-parametric entrainment, adequate to explain the facts

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in the pupal eclosion rhythm of *Drosophila* (Pittendrigh, in preparation; Ottesen et al., in preparation) had wider utility. Could it account equally well for the entrainment of circadian pacemakers in vertebrates? Nocturnal rodents seemed appropriate experimental animals in pursuing this question: their entrainment in nature may depend on the interaction of two major light signals each day – one at dawn when they retreat to dark nests and a second near sunset when their major activity begins.

The essential elements in the non-parametric model are two properties of the pacemaker: its freerunning period (τ) and phase-response-curve (PRC) for brief light pulses. In the *Drosophila* case the success of the model derives in part from the considerable precision with which both parameters can be measured experimentally. This precision is probably due to the fact that the assay of both τ and PRC for an eclosion rhythm yields average values for both parameters based on the behaviour of hundreds of individual pacemakers. There are substantial difficulties in attempting to estimate the real variance on both τ and the PRC shape of an individual *Drosophila* pacemaker, and we were, therefore, uncertain about what to expect in using techniques based on individual rodents. A major part of our work has therefore addressed that issue: what is the magnitude of both inter- and intra-individual variation in τ and PRC? To what extent can the variation encountered be traced to systematic sources and accounted for in an analysis of entrainment?

The four species of nocturnal rodents we chose are well-known for the precision with which they express their circadian rhythm of running-wheel activity. For one of them, the golden hamster, phase response curves of single animals were already available (Burchard, 1958; DeCoursey, 1964). The long-term nature of the experiments involved in this study (Table 1) is relevant to some of its shortcomings. The importance of some variables and questions emerged only gradually with the consequence that some experiments (too long to repeat lightly) lack the design we now wish they had had. Nevertheless, the facts available lead to a coherent picture: the non-parametric model developed for *Drosophila* is, in general, applicable to nocturnal rodents. That is not to say it explains all the facts; but where it has fallen short it has served, heuristically, to focus attention on issues that might otherwise have remained unnoticed. More generally it has provided a conceptual framework without which we would have failed to find meaning in several of the new empirical regularities we report.

It is worth noting that circadian rhythm research has so far involved little comparative physiology of related species, and that we have found the comparison of 4 different nocturnal rodents, including 2 in the same genus, a valuable approach. The empirical regularities we report in later papers concerning the interdependence of the pacemaker's circadian period (τ), its lability, and the shape of its PRC were first encountered in the interspecific comparison which then prompted their discovery in differences between individuals within the species and, to some extent, within the individual animal itself. The generality of their interdependence (τ , τ -lability, and PRC shape) must have meaning which we have sought in two different directions.

First, it has led us to a formulation of the issues involved in the adaptive strategies open to natural selection in evolving peculiarities of circadian pace-

Table 1. Types of experiments and numbers of animals involved. Roman numerals refer to papers in this series where the experiments are discussed

Description	Year	Experimenters and technical assistance	<i>M.</i> <i>musculus</i>	<i>P.</i> <i>leucopus</i>	<i>P.</i> <i>maniculatus</i>	<i>M.</i> <i>auratus</i>
After-effects of T (I)	1962	PB, PK	33			
After-effects of photoperiod (I)	1962/63 1974	GD, DI, DP, JSch.	10	7		10
After-effects of skeleton photoperiod (I)	1974/75	DP	13			
After-effects of LL (I, V)	1964/1965	KA, JS		8		12
Age effects (I)	1965/66 1974	KA, JS, DP	14	8	9	8
Behaviour in LL (V)	1964/67	KA, JS				39
τ (light intensity) (III)	1973	FD		8	7	5
Phase response curves (II)	1966	KA, JS		5	7	13
Phase response curves in H ₂ O, D ₂ O (II, III)	1974	JSch.	32			
τ (D ₂ O) (III)	1973/75	RB, MS, DP	21	4	9	4
Phase shifts during after-effects (II)	1974/75	PS, DP	34			
Entrainment with different T (IV)	1965/66	KA, JS		3	6	31
Skeleton photo- periods (IV)	1963/64	KA		19		19

makers as “biological clocks”: not only to measure the lapse of time (as in sun-compass orientation), but more generally to recognize local time. The latter function involves maintenance of a stable phase-relation between the circadian pacemaker and the external cycle of light and darkness that entrains it. The challenges here derive not only from some inherent instability of pacemaker frequency, but, more importantly, from the regular seasonal change in a major feature (photoperiod) of the entraining agent. The fact that the pacemaker's average period ($\bar{\tau}$) is sometimes set well away from 24 h, and the interdependence of $\bar{\tau}$, τ lability, and PRC shape find functional meaning in this context. Second, the generality of that interdependence has led us to seek a maximally simple formal model of the pacemaker capable of yielding the behaviour involved.

The first paper addresses the questions: how stable is τ ? What is the magnitude of its random, day-to-day, variation? And what, if any, are the systematic sources of τ -variation?

We wish to acknowledge the extremely valuable technical assistance and collaboration of many individuals in the maintenance of animals, in their experimental manipulation, and in the arduous task of data reduction. These include, at Princeton: Kenneth Arnott, Paul D. Bostrom,* George T. Dewey,* Dean M. Ishiki,* Peter S. Kennedy,* Lawrence A. Plumlee,* and Johanna Snickers; at Stanford: Robert Benedetti, Fred Davis,* Gary Domingos, Diana Page, John Schoenberger, Marilyn Sigman, and Peggy Sonnenschein. Several of these collaborators, asterisked above, were undergraduate students whose contributions to our program provided the material for research reports. The experiments to which they contributed are noted in Table 1.

One of us (C.S.P.) continues to be greatly indebted to his former Princeton colleagues, Victor G. Bruce and Dorothea M. Minis, for valuable discussion and constructive criticism in the development of the work. The work was supported by funds from the National Aeronautics and Space Administration, The National Institute of Mental Health, and The Whitehall Foundation. S.D. was supported by a fellowship from the Netherlands Organisation for the Advancement of Fundamental Research (Z.W.O.).

II. Materials, Methods, and Terminology

a) Assay of Activity Rhythms

The rodents studied were: Golden hamster, *Mesocricetus auratus*; whitefooted mouse, *Peromyscus leucopus*; deer mouse, *Peromyscus maniculatus*; and house-mouse *Mus musculus* (C57BL and DBA strains). Hamsters were obtained from a local animal dealer; *Peromyscus maniculatus bairdii* was obtained from the Jackson Laboratories in Bar Harbor, Maine; *P. leucopus novaborencensis* was trapped locally in Princeton, New Jersey. *Mus musculus* were obtained from Simonson Laboratories in Gilroy, California and the Charles Rivers colony in Boston, Massachusetts. Only males were used in all species. Part of the experimental animals were born and raised from stocks in the laboratory.

The circadian rhythm of running-wheel activity was assayed in all animals. Each was housed in a cage (7' × 13" × 9" high) inside an individual light-tight box (15' × 21" × 18" high). The boxes were ventilated through light-traps. Each box contained an overhead 4-Watt white fluorescent lamp in a water jacket, with continuous water flow to eliminate temperature change when light signals were given. The ambient temperature was 20 °C. Rotation of the running wheel in each cage activated a microswitch coupled to an operations recorder (Esterline-Angus). As many as 80 animals in separate boxes were assayed concurrently.

The light regime in each box was independently clock controlled. Each animal could be maintained, independently of others, in constant darkness (DD), constant white light (LL), or a light-dark (LD) cycle in which both the duration of the light, and the dark period could be manipulated. Single light-pulses could also be administered (via clock control) to animals free-running in DD. The light intensity was between 100 and 200 Lux unless otherwise specified. In some instances it was reduced to lower intensities using large neutral density filters over the aperture of the overhead light box. Food and water checks of animals in a DD schedule were always done in dim red light (>610 nm), to which the circadian rhythms appeared to be insensitive.

The raw data from the Esterline Angus operations recorder were pasted day for day onto standard charts, then photographically reduced and "double-plotted" in the now standard way illustrated in Figure 1. In later figures we have adopted the custom of indicating the light-dark treatment in the right half of the figures only and showing the unedited raw data in the left half.

b) Estimation of Parameters of the Rhythm

The "freerunning period" (τ) of the activity rhythm in constant conditions was estimated by two techniques. The estimate was always based on the onset of activity, which, in the great majority of records, is a more precise "marker" of the rhythm than the end of activity. In some instances, we have calculated the linear regression through successive daily onsets of activity. In other cases, the slope of an eye-fitted line through the onsets was used. The first 2-4 days of a freerun following a resetting light pulse or a change in conditions are often characterized by transient phase-shifting and these onsets are then left out of account in the determination of the freerunning period. We have made an empirical study of the reliability of estimating τ using eye-fits. Five people independently fitted lines through the activity onsets of 86 different freeruns of *Mus musculus*, for which τ had been calculated by linear regression. 95% of the 430 eye-fit estimates were different from the regression estimate by less than 0.12 h. Eye-estimates were slightly better for long τ 's ($23.0 < \tau < 24.0$; 95% of 325 estimates within 0.10 h) than for short τ ($\tau < 23.0$; 95% of 105 estimates within 0.14 h). For most purposes the accuracy of eye-fits is obviously adequate.

The fraction of each cycle devoted to running wheel activity is designated α ; ρ designates the rest fraction (Fig. 1). The value of α is the interval (in hours) between eye-fitted lines marking onsets and cut-offs of activity. It is a less precisely measured parameter of the cycle than τ because of the poor definition of cut-offs compared to the onsets on which τ is based.

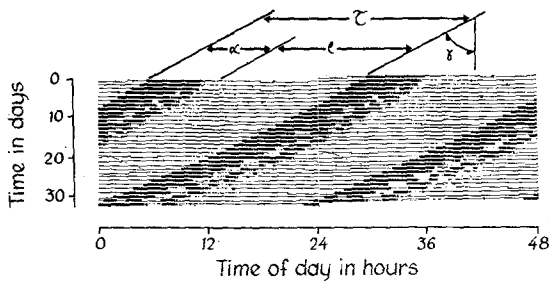


Fig. 1. Parameters of the freerunning activity rhythm in DD. The period (τ) is calculated from the slope ($\tau = 24 \text{ h} - \text{tg } \gamma$) of a line fitted by eye or by linear regression to a series (usually 10) of onsets of activity. τ is divided in activity time (α) and rest time (ρ). For continuity, the record (*Peromyscus leucopus* # 1106) is shown twice

In discussing the successive phases through which the rhythmic system passes in each cycle, we will use the terminology of *circadian time* introduced by Pittendrigh and Minis (1964). The full circadian cycle (360°) is considered to last 24 circadian hours, one circadian hour lasts $\tau/24 \text{ h}$ of real time. The successive phases (ϕ) can be called circadian times (ct): ϕ (in degrees of arc) and ct (in circadian hours) are equivalent notations. In the entrained steady-state established by a light/dark cycle of 12 h of light and 12 h of darkness (LD 12:12), that part of the rhythm which falls in the light is designated the “subjective day”; the part falling in the dark is the “subjective night.” In our rodents the onset of running-wheel activity typically begins at or near the onset of dark in LD 12:12 and we, again arbitrarily, designate that phase (activity onset) as ct 12: it is the most reliably assayed phase and other ct points are computed by reference to it.

III. Results

a) Interspecific and Interindividual Variation in τ

Figure 2 summarizes the gross variation in τ in constant darkness (DD) in the four rodents studied. It includes 1719 estimates of τ each based on a section of a freerun of at least 10 cycles. The values range from 21.8 to 25.5 h. While those minimal and maximal values were both found in the same species (*P. maniculatus*) there are obvious interspecific differences both in τ and its variability. Two species (*M. auratus* and *P. leucopus*) have $\bar{\tau}$ (the species average) indistinguishably close to 24 h; while $\bar{\tau}$ in *M. musculus* and *P. maniculatus* is about half an hour shorter. The raw facts in Figure 2 already indicate the important fact, which is treated more fully later, that the variability of τ is smaller in the species with $\bar{\tau}$ closer to 24 h.

Subsequent sections show that τ for any given 10-day section of a freerun is history-dependent. Figure 2 pools all observations for the four species and makes no allowance for differences between them in the extent to which they experienced different treatments prior to the measured freerun known to affect τ . The distribution in *Mus musculus*, for example, must be significantly affected by inclusion of many values obtained from experiments in which τ was deliberately shortened and lengthened by prior exposure to very short and very long light cycles (see Fig. 8). Figure 3, involving smaller sample sizes, is probably a better reflection of

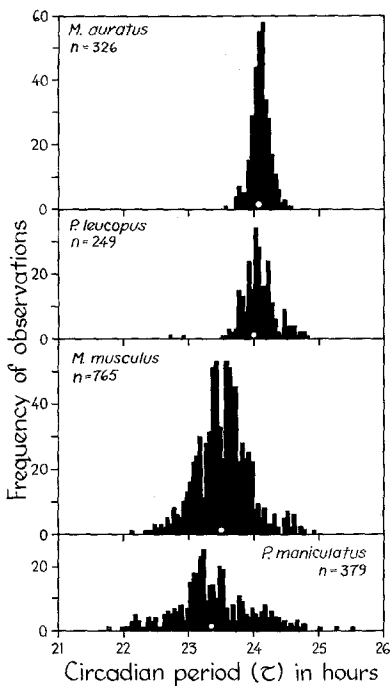


Fig. 2. Histograms of all measurements of τ (in 3' bins) in constant darkness (DD) made in analyzing the experiments in this and subsequent papers. Each observation is based on at least a 10-day segment of a freerun. For many animals more than one observation is included. Open circles are the means of the distributions

inter-specific differences in both pacemaker period ($\bar{\tau}$) and its inherent lability. These τ -estimates were all derived from animals freerunning for months in DD after prior entrainment to LD 12:12. They were, however, being subjected to small phase-shifts every two weeks in experiments from which phase response curves were derived (see Daan and Pittendrigh, 1976a). Such phase-shifts have small after effects on τ (Table 4) but these are randomly distributed among the animals on which Figure 3 is based. The species differences in both $\bar{\tau}$ (i.e. individual average) and its variance are even clearer now; so is the dependence of its variance on the proximity of $\bar{\tau}$ to 24 h. The figure also shows that individuals within a species may have significantly different $\bar{\tau}$'s. The difference in $\bar{\tau}$ between two individual hamsters may exceed the difference in $\bar{\tau}$ between *M. auratus* and *P. leucopus*. There is a clear trend, comparing the four species, that as $\bar{\tau}$ gets further away from 24 h the standard deviation on $\bar{\tau}$ increases. In those species (*P. maniculatus* and *M. musculus*) where the intraspecific range of $\bar{\tau}$ variation is large enough, the same trend is indicated among individuals (see also Pittendrigh and Daan, 1976a, Fig. 21).

b) Intra-individual Variation: Pacemaker Versus Overt Rhythm

There is still, in our view, insufficient attention given to the distinction that should be made between the properties of a directly observable circadian rhythm and those of the pacemaker that drives it (Pittendrigh, 1967; in preparation): to what

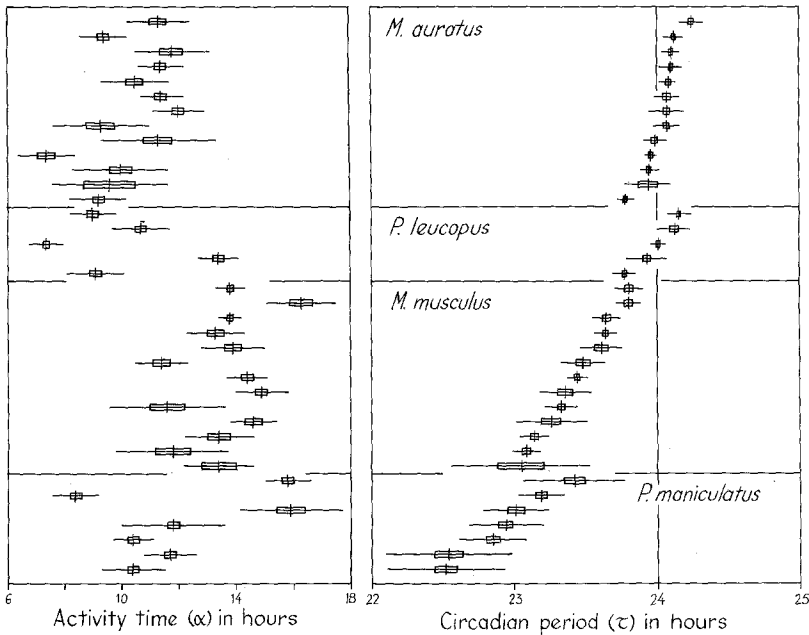


Fig. 3. Variation in activity time (α) and freerunning period (τ) of the circadian activity rhythm of four species of rodents. Vertical lines indicate mean α and mean τ for single individuals, ± 1 standard error (rectangles) and ± 1 standard deviation (horizontal lines). The results are based on prolonged freeruns interrupted by single light pulses every two weeks (Daan and Pittendrigh, 1976b); measurements of α were obtained by eye-fit, measurements of τ were obtained by linear regression over 10–14 cycles

extent do the statistical properties of the driven rhythm reliably reflect those of its pacemaker?

Figure 1 records the activity of a white footed mouse freerunning in constant darkness for 32 days. The onsets of activity shift progressively forward, with remarkable precision. If we tabulate the times of activity onset over a section, e.g., the first ten days, of this freerun, we find a value for τ of 23.19 h, whether measured as the average interval between the onsets or from the linear regression line through them. The standard deviation of the intervals is 0.08 h. Surely, we must take 23.19 h, measured from the rhythm, to reflect accurately τ of the pacemaker. But it is not clear that the standard deviation of 0.08 h is a reliable estimate of the day-to-day variation in pacemaker periods. A first indication that it is not, is found when we compute Pearson's coefficient of correlation between successive intervals. Over the same section of the run, this "serial correlation coefficient" (r_s) has a value of -0.47 . This indicates that if one interval (t_i) between two onsets exceeds 23.19, the next interval (t_{i+1}) has a probability greater than 0.5 of being less than the average. Something constrains the sign of successive periods of the rhythm. One may visualize the issues involved in several equivalent ways; one is to assume that at some standard phase in its motion the pacemaker triggers a train of physiological events that culminate in activity onsets, which is what we observe. The total variance on the periods of successive onsets then comprises

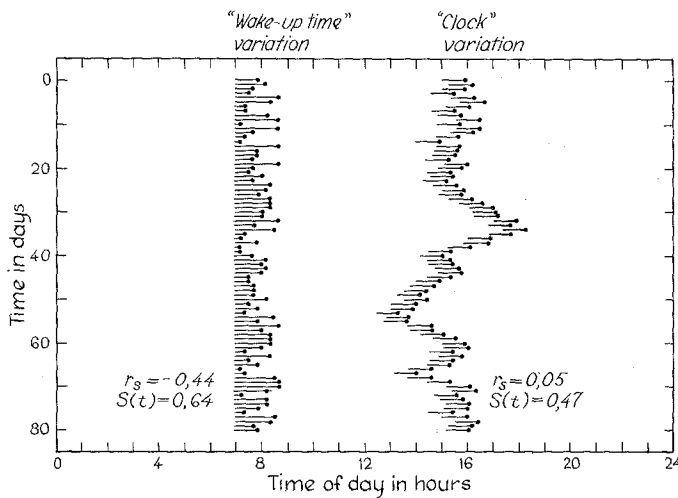


Fig. 4. Simulations showing contributions of hourglass processes and pacemaker to the observed variation in an overt circadian rhythm. In one simulation ("wake-up time" variation), clock periods were kept constant at 24.0 h and random variations taken from a table of random numbers, introduced into ancillary processes (horizontal lines) leading to activity onset (dots). In the second simulation ("clock" variation) identical variations were introduced into the pacemaker periods and the ancillary processes kept constant at 55 min. r_s = coefficient of serial correlation between consecutive periods ($t_i, t_i + 1$) of the overt rhythm. $s(t)$ = standard deviation of these periods

two distinct components. One is variance on the pacemaker's period; the second is variance on the duration ("wake-up time") of those processes which, having been initiated by the pacemaker, end in the onset of activity.

In Figure 4 two simulations are shown of an activity rhythm with an average period of 24 h and an average "wake-up time" of 55 min. In the first simulation, we have kept the clock period constant and added random variations to the wake-up time (from -45 to $+45$ min), taken from a table of random numbers. In the second simulation, exactly the same variations were added to the clock period, with wake-up time held constant. It is clear that the two sources of variation contribute in a radically different way to the observed rhythm. Wake-up time variation is like the non-genetic component in the phenotypic variability of successive generations: it is not transmitted from one generation to the next. On the other hand when wake-up time variation is eliminated completely, our simulation parallels that of random drift in gene frequency in successive generations.

If we look at observed *periods* (the time interval t_i between one activity-onset and the next) in the two simulations we find a major difference in the serial correlation coefficient (r_s) which estimates the degree of correlation between successive values of t_i . When all the variance is attributable to the pacemaker ("clock") the coefficient is close to zero (0.05); when all the variance is attributable to wake-up time it is negative (-0.44). Apparently the serial correlation coefficients (r_s) derived from real data on successive periods (t) of the observed rhythm can tell

us something about the relative contributions of pacemaker variations (day-to-day variations on τ_i) and wake-up time variation.

If pacemaker periods are denoted as τ_i , wake-up times as w_i , and observed periods of the rhythm as t_i , it is clear that

$$t_i = \tau_i - w_i + w_{i+1}$$

and

$$t_i + t_{i+1} = \tau_i + \tau_{i+1} - w_i + w_{i+2}.$$

Independent variations in w and τ would then lead to:

$$\sigma^2(t) = \sigma^2(\tau) + 2\sigma^2(w)$$

and

$$\sigma^2(t_i + t_{i+1}) = 2\sigma^2(t) + 2\text{cov}_s(t) = 2\sigma^2(\tau) + 2\sigma^2(w),$$

where $\text{cov}_s(t)$ indicates the covariance of successive observed periods of the rhythm.

Hence,

$$\sigma^2(w) = -\text{cov}_s(t)$$

and

$$\sigma^2(\tau) = \sigma^2(t) - 2\text{cov}_s(t).$$

If in a sample $\text{cov}_s(t)$ is estimated by $r_s \cdot s(t)$, with $s(t)$ denoting the standard deviation of t , then the variances of w and τ are estimated by:

$$s^2(w) = -r_s \cdot s^2(t)$$

$$s^2(\tau) = (1 + 2r_s) \cdot s^2(t)$$

In the examples of Figure 2, we get for the first case

$$s^2(w) = +0.44 \times 0.64^2 = 0.18, \quad s(w) = 0.42$$

$$s^2(\tau) = (1 - 0.88) \times 0.64^2 = 0.15, \quad s(\tau) = 0.22$$

and for the second case:

$$s^2(w) = -0.03 \times 0.47^2 = -0.007, \quad s(w) = \sim 0$$

$$s^2(\tau) = (1 + 0.06) \times 0.47^2 = 0.23, \quad s(\tau) = 0.48$$

This partitioning of the variance, which was outlined for us by Dr. Roger Pinkham, can only be applied when the successive clock periods and wake-up times are not correlated. Consequently only relatively short runs can be used in which the average period of the circadian rhythm is apparently not changing. The serial correlation coefficient $r_s(t)$ was calculated for 90 short (10–15 days) DD-freeeruns in an experiment with *Mus musculus* which is described elsewhere (Daan and Pittendrigh, 1976a). The majority of values was negative (Fig. 5). The average of the distribution is -0.36 . This would indicate that, on the average

$$s^2(w) = 0.36 \cdot s^2(t) \quad \text{or} \quad s(w) = 0.60 \cdot s(t)$$

and

$$s^2(\tau) = (1 - 0.72) \cdot s^2(t) \quad \text{or} \quad s(\tau) = 0.53 \cdot s(t)$$

The average standard deviation of the observed periods $s(t)$ was 0.282 h (1.2% of $\bar{\tau}$), giving us overall estimates for wake-up time variation:

$$s(w) = 0.60 \times 0.282 = 0.169 \text{ h}$$

and for clock variation:

$$s(\tau) = 0.53 \times 0.282 = 0.149 \text{ h} (=0.6\% \text{ of } \bar{\tau}).$$

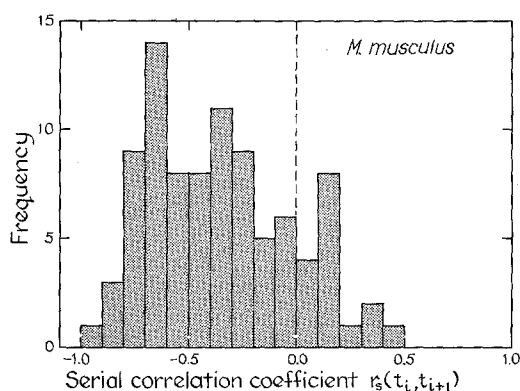


Fig. 5. The distribution of calculated values of the serial correlation coefficient r_s in 90 short (10–15 days) DD-freeruns in *Mus musculus* C57

Our conclusion is, therefore, that variability of the clock period and variability in the aperiodic processes called wake-up time contribute about equally to the cycle-to-cycle variations in the observed rhythm. Further, we judge that the precision of the circadian pacemaker in *Mus musculus* is on average twice as good as the precision of the observed activity rhythm. The same data from *Mus musculus* allow us to compare “precise” [$s(t) \leq 15$ min] and “sloppy” rhythms [$s(t) > 15$ min]. As shown in Table 2 the mean serial correlation coefficient (r_s) was -0.292 (\pm s.e. 0.054) for 42 “precise” runs and -0.412 (\pm s.e. 0.045) for 48 “sloppy” runs. The difference is highly significant. It turns out that the difference in the rhythm’s precision is attributable, almost entirely, to difference in wake-up time variation; the precision of the pacemaker is about the same in both groups (standard deviations of 0.102 and 0.109 h, or about 6 min). In Table 2 some estimates of r_s are included for the other species: they show that again the values are negative and indicate contributions of both clock and wake-up time variation to the observed rhythm. The runs analyzed are too few to attach more importance to the partitioning here.

Our conclusion is that the day-to-day variation in the directly observed period of a circadian rhythm is not, of itself, a reliable guide to the instability of the pacemaker driving that rhythm: “sloppy” activity records may occur in spite

Table 2. Partitioning of the variance on the observed circadian periods in rodents: \bar{r}_s = average serial correlation coefficient; $\bar{s}(t)$ = average standard deviation of intervals between onsets of activity; $\bar{s}(w)$ = estimated average s.d. of “wake-up times”; $\bar{s}(p)$ = estimated average s.d. of pacemaker periods (see text)

Species	Nr. of animals:	Number of runs	\bar{r}_s	$\bar{s}(t)$	$\bar{s}(w)$	$\bar{s}(p)$
<i>M. musculus</i>	$s(t) < 0.25$	15	42	-0.292	0.158	0.085
	$s(t) > 0.25$	18	48	-0.412	0.395	0.254
	All runs	20	90	-0.356	0.282	0.169
<i>P. leucopus</i>		8	15	-0.375	0.328	0.201
<i>P. maniculatus</i>		2	16	-0.255	0.614	0.310
<i>M. auratus</i>		2	15	-0.264	0.135	0.069
				h	h	h

of a precise pacemaker and the latter usually appears to be more precise than the raw data suggest.¹ On the other hand, given the large range of variations in r_s itself, documented in Figure 5, it is clearly not possible to estimate pacemaker precision itself with any accuracy from single runs. Because one cannot routinely undertake the large number of separate analyses summarized for *Mus musculus* in Table 2, estimates of day-to-day variation in pacemaker performance will always be overestimates, with the actual precision being masked to some extent by an overlay of some unknown extraneous source of variance ("wake-up time"). On the other hand both sources of variance play a decreasing role in our estimate of τ the more cycles are included in the estimate.

Henceforth in this paper when we talk about lability of the pacemaker we will always mean variation on estimates of τ from different freeruns (of at least 10 cycles).

c) Intra-individual Variation: The History Dependence of τ

There is a general *lability* of pacemaker period that is to be distinguished from its day-to-day *instability*. This lability of τ is not purely random: it reflects an inertial aspect of the pacemaker which tends to preserve a state, characterized by τ , into which it has been forced by entraining agents. This feature of circadian pacemakers was initially reported by Pittendrigh (1960) who described the phenomena as "after-effects". He found that when a large light pulse imposed a major phase-advance on a hamster rhythm freerunning in darkness the new steady-state was characterized by τ shorter than it had been prior to the phase-shift. A phase-delay lengthened τ .

In systems where the duration of a freerun is limited by inexorable biological reasons to a relatively few cycles (*Drosophila pseudoobscura* and the moth *Pectinophora gossypiella*) it is a problem to distinguish confidently and meaningfully between transients and after-effects. In the circadian literature the term "transient" has been given its own special meaning (Pittendrigh et al., 1958; Pittendrigh, 1967) different from the mathematician's: a circadian transient is a cycle of the system's motion with a period longer or shorter than that of the steady-state τ ; it occurs during the transition from one steady-state to another. Thus an advance phase-shift ($+\Delta\phi$) is effected by the occurrence of one or more transient cycles each of which has a duration shorter than τ ; a delay phase-shift ($-\Delta\phi$) is effected by one or more transient cycles each lasting longer than τ . In *Drosophila pseudoobscura* there are after-effects on τ of constant light, of T , of a phase-shift and of photoperiod duration in a light cycle with $T=24$ h (unpublished observations); but since the population eclosion rhythm can rarely be followed for more than 10 cycles are we not merely describing, in new words, the fact that there are long-lasting transients imposed by these prior conditions?

Figure 6 gives an initial justification for maintaining that *transients* and *after-effects* can be usefully distinguished. It summarizes the behaviour of 8 hamsters

¹ As Dr. J. T. Enright has pointed out to us, some of this extra variation is not merely spontaneous, but is related to identifiable exogenous factors. Feeding disturbances, for instance, sometimes cause a 'once-only' delay in the onset of activity (see, for example, the *Glaucomys volans* activity record published by DeCoursey, 1961, Fig. 1)

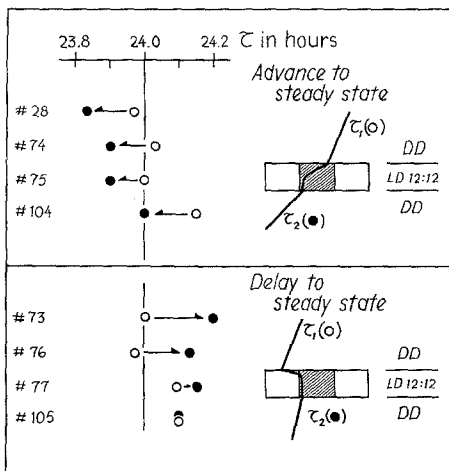


Fig. 6. After-effects of transients in hamsters (*M. auratus*). The freerunning period (τ) was measured in all 8 animals before (\circ) and after (\bullet) a 21-day period of entrainment to LD 12:12. Four of the animals (upper panel) phase-delayed to the entrained steady-state; the other four (lower panel) phase-advanced to the entrained steady-state. In 7 out of the 8 animals τ in the second freerun shows an after-effect of the sign of the phase shifts by which the brief steady-state was approached. (Data redrawn from Pittendrigh 1960, Fig. 7)

whose freerunning rhythm in DD was interrupted by a brief (21 day) period of entrainment to an LD 12:12 light cycle. The phase (relative to local time) of the initial freerun was different in each animal. All were then exposed to a daily 12 h photoperiod beginning at the same local time. All entrained to the light cycle, reaching an apparent steady-state in about a week. The initial phase of four animals was such that the light cycle delayed them to the entrained steady-state; in the others the steady-state was reached by advances. After apparently stable entrainment by the light cycle for at least 2 weeks, the period of the subsequent freerun showed an after-effect of the transient motion that brought them into this steady-state. It seems pointless here to insist that the *transients* were not over; the entrained system manifested overt stationarity with a 24 h period for many cycles. What the experiment reveals is an inertial effect or a history-dependence: both freeruns, before and after the intervening entrainment, give every usual indication of being a stationary periodicity.

We recognize that transients and after-effects may be quantitatively different expressions of a qualitatively similar, or identical, pacemaker property (Pittendrigh and Daan, 1976b). But the difference is so great it is useful to distinguish them with the following operational definitions: *Transients* are cycles of rapidly changing duration intervening between two steady-states, each characterized by a nearly stable τ . The two steady-states are typically separated by no more than 10 transients—sometimes by only one; *after-effects* are long-lasting, slowly decaying, changes in pacemaker period incurred by prior experience. In a subsequent section we report the endurance for about 100 cycles of after-effects on τ in *Mus musculus*. But at any time during the slow decay of those after-effects the pacemaker can be rapidly re-entrained by a 24 h zeitgeber requiring only a few *transients*.

The literature has paid little attention to after-effects in the 15 years since they

Table 3. After effects on τ in constant darkness

Prior Conditions Species	Sign of the after-effect ^a		Reference
I. Phase-shift (in a freerunning rhythm)	$+\Delta\phi$	$-\Delta\phi$	
<i>Mus musculus</i>	—	+	this paper, Table IV
<i>Mesocricetus auratus</i>	—	+	Pittendrigh (1960)
<i>Peromyscus leucopus</i>	—	+	this paper, Table IV
<i>Taeniopygia guttata</i>	—	+	Pittendrigh (1960)
<i>Passer domesticus</i>	—	—	Eskin (1971)
<i>Drosophila pseudoobscura</i>	—	+	Pittendrigh (unpubl.)
<i>Ammospermophilus leucurus</i>	0	—	Kramm (1971)
II. Entrainment (effect of T)	$T < \bar{\tau}$	$T > \bar{\tau}$	
<i>Peromyscus leucopus</i>	—	+	this paper, Fig. 10
<i>Mesocricetus auratus</i>	—	+	this paper, Fig. 9; Pittendrigh (1960, 1974)
<i>Mus musculus</i>	—	+	this paper, Figs. 7, 8
<i>Passer domesticus</i>	—	+	Eskin (1971)
<i>Drosophila pseudoobscura</i>	—	+	Pittendrigh (unpubl.)
<i>Pectinophora gossypiella</i>	—	+	Pittendrigh (unpubl.)
<i>Leucophaea maderae</i>	—	+	Caldarola (unpubl.)
III. Constant light			
<i>Mesocricetus auratus</i>	0		this paper, Fig. 11
<i>Peromyscus leucopus</i>	+		this paper, Fig. 11
<i>Drosophila pseudoobscura</i>	+		Pittendrigh (unpubl.)
IV. Entrainment (effect of photoperiod in $T = 24$ h)	PP short	PP long	
<i>Peromyscus leucopus</i>	+	—	this paper, Figs. 12, 13; Pittendrigh (1974)
<i>Mesocricetus auratus</i>	0	0	this paper, Fig. 13
<i>Myotis lucifugus</i> ^b	+	—	Menaker (1961)
<i>Passer domesticus</i>	+	—	Hudson and Kimzey (1966); Eskin (1971)
<i>Zonotrichia leucophrys</i>	—	+	Gwinner (1975)
<i>Zonotrichia atricapilla</i> ^c	—	+	Gwinner (1975)
<i>Carduelis flammea</i> ^{b,c}	+	—	Pohl (1972)
<i>Drosophila pseudoobscura</i>	—	+	Pittendrigh (unpubl.)

^a + : induces increase in τ ; — : induces decrease in τ ; 0: no effect^b Prior condition: natural photoperiod in summer and winter^c in dim LL (0.02 Lux)

were first reported, as Table 3 indicates; but that table also shows they are widespread. All known after-effects are attributable to light, the principal entraining agent of the pacemaker.

d) After-Effects of Phase-Shifts

Changes in τ due to a single large phase shift, induced by a long (e.g. 12 h) light signal, have been reported in several species (Pittendrigh, 1960; Eskin, 1971).

Table 4. After-effects of phase shifts: average changes ($\overline{\Delta\tau}$) of τ in hours, with standard errors (s.e.) and number of observations (n), related to advance phase shifts ($\Delta\phi > 10^\circ$) and delay phase shifts ($\Delta\phi < -10^\circ$). p indicates the significance level of the difference of the means of both groups (t -test)

Species	Advance Phase Shifts			Delay Phase Shifts			p
	$\overline{\Delta\tau}$	s.e.	n	$\overline{\Delta\tau}$	s.e.	n	
<i>M. auratus</i>	-0.040	0.009	50	-0.010	0.016	23	<0.1
<i>P. leucopus</i>	-0.059	0.017	8	0.011	0.016	12	<0.01
<i>P. maniculatus</i>	-0.007	0.025	26	0.002	0.019	51	>0.1
<i>M. musculus</i>	-0.108	0.031	13	0.033	0.030	38	<0.02

Smaller phase shifts, themselves caused by smaller (15 min) light signals produce less clear and consistent after-effects on τ ; but they do occur. Table 4 is based on data analyzed in deriving the phase response curves we discuss in a later paper (Daan and Pittendrigh, 1976a). It shows a relationship of the average change in τ , between two 14 day freeruns separated by a single light pulse, with the sign of the phase shift produced by that pulse. Delay phase shifts ($\Delta\phi < -10^\circ$) were, except in the hamster, associated with average increases in τ , advance phase shifts ($\Delta\phi > 10^\circ$) with decreases in τ . In *P. leucopus* and *M. musculus* the difference between the sample means is statistically significant. The effect is consistent among six different species (see Table 3), although Kramm (1971) found decreases in τ following delay phase shifts in the antelope ground squirrel. Whether or not the opposite effect relates to the diurnality of his rodent remains to be clarified.

e) After-Effects of Entrainment

If the entrainment of the pacemaker reduces to a succession of phase-shifts that compensate for the difference between its period (τ) and that (T) of the entraining cycle (Pittendrigh, in preparation; Ottesen et al., in preparation), we must expect that entrainment itself entails after-effects; and it does. The effect is clearest, certainly most dramatic, following entrainment by exotic light-cycles.

Figures 7 and 8 record the effect on *Mus musculus* of light cycles much shorter, and much longer, than 24 h. After initial entrainment to either LD 11.5:11.5 ($T=23$) or LD 12.5:12.5 ($T=25$) the length of each cycle was decreased or increased by 10 min each day until, after 18 days the period (T) of the light cycle was ca. 20 h in the short T group and ca. 28 h in the long T group. The majority of animals continued to follow the light cycle until they were released to free-run in constant darkness. Some of them lost entrainment in the extreme long and short cycles. In those animals released from $T=28$, the average initial value of τ (in the first 10 days of the freerun) was 24.36 h; while in those released from $T=20$ the average initial value of τ was 23.12 (Fig. 8, right panel). These values are larger and smaller respectively than either of our estimates of $\bar{\tau}$ (from Figs. 2 and 3) for *M. musculus* (23.50 and 23.43). The large sample sizes leave no doubt of the significance (t -test, $p < 0.001$) of the effect. Moreover, it is clear that this induced change in τ in the two groups decayed only very gradually; it is still significant ($p < 0.01$)

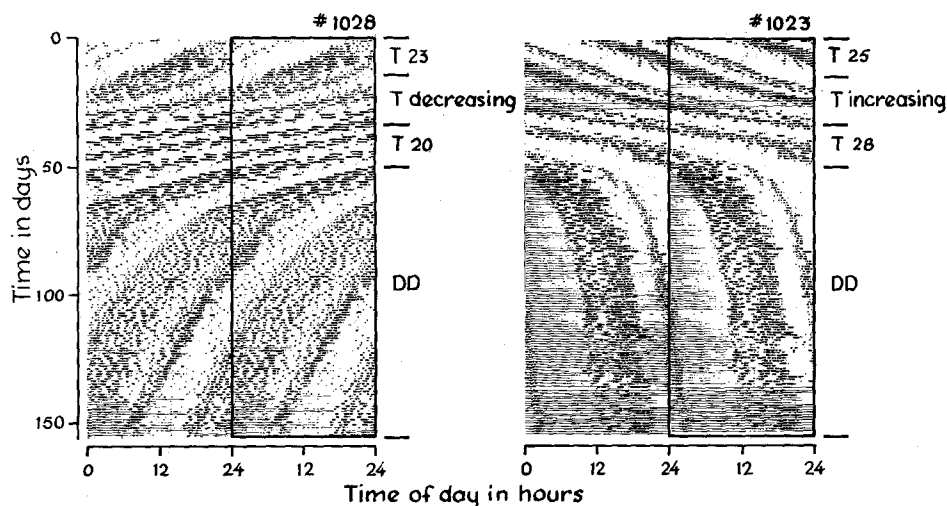


Fig. 7. Examples of after-effects of T in *Mus musculus*. Entrainment of the animals to the extreme light-cycles LD 10:10 (T 20) and LD 14:14 (T 28) was achieved by gradually decreasing T by 10'/day from T 23 and T 25 respectively. Upon release into DD, the animals only gradually returned to τ -values closer to 24 h. The animal (# 1028) entrained to short T in DD retained a smaller τ than the animal (# 1023) entrained to long T

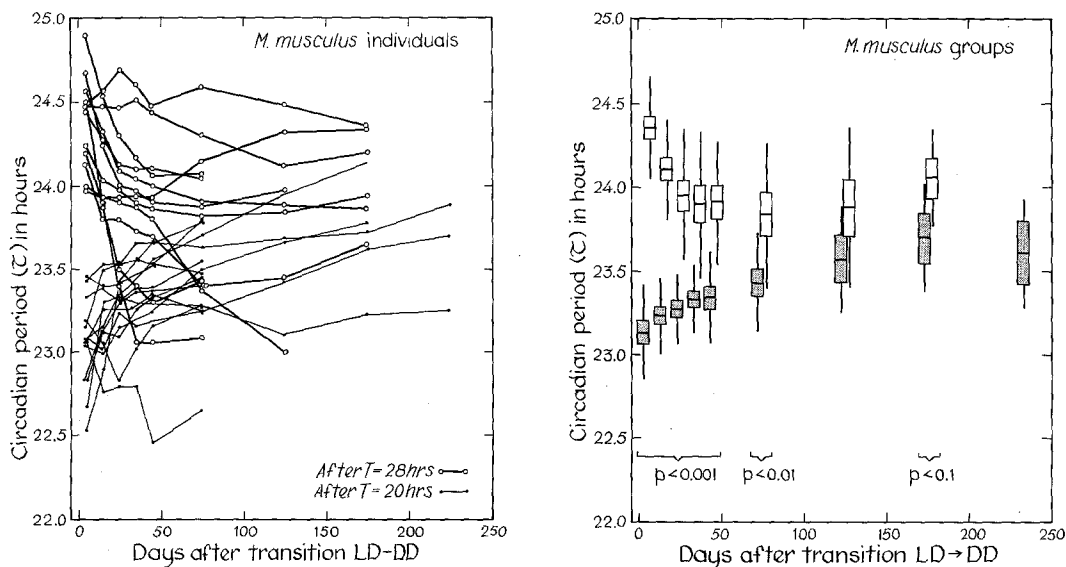


Fig. 8. After-effects of previous entrainment to short and long light-dark cycles in *Mus musculus*. In the left panel individual τ -values measured over 10-day intervals (days 1–50) and over 50-day intervals (days 50–250) are shown. Mice released from LD 10:10 are represented by dots, those released from LD 14:14 by circles. Only the 26 animals with freeruns of at least 100 days are shown. In the right panel average τ values, standard errors (rectangles) and standard deviations (lines) are shown for the two groups. All 33 animals involved in the experiment, six of which were tested with both prior treatments, are included. Significances of the differences of the means between the two groups are based on a t -test, and are indicated in the lower part of the panel

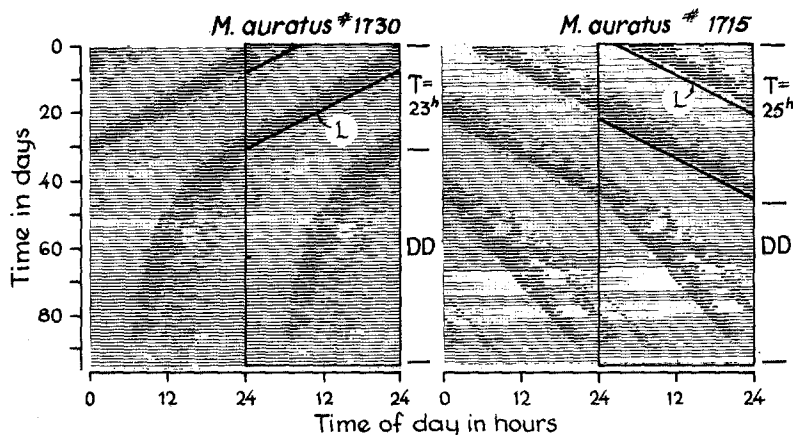


Fig. 9. Examples of after-effects of T in the golden hamster. After prolonged entrainment to T 23 and T 25 (15' light per cycle), animals released from T 23 to constant darkness (DD) tended to run free with τ less than 24 h (# 1730), those released from T 25 with $\tau > 24$ h (# 1715). Notice further (1) the difference in ψ between the two animals during entrainment. In T 23, the light pulse hits the rhythm at the end of activity, in T 25 at the onset of activity. (2) The difference in α between entrained and freerunning steady state. In both T 23 and T 25 α is shorter during entrainment than in the subsequent freerun

after 50–100 cycles. By that time the two groups had converged on a value of τ less than 24 h, which in some sense must represent the average “natural” – or “most probable” (τ_p) – state of the *M. musculus* pacemaker in constant darkness. The left panel of Figure 8 gives the time-course of the decay of the after-effects in the 26 animals in which the freerun lasted at least 100 cycles. The inter-individual variation is clear: the “most probable” state (τ_p) is different among individuals.

Comparable effects of zeitgeber periods far from 24 h have already been reported for hamsters by Pittendrigh (1974). In Figure 9 the initial value of τ in the animal released from $T=23$ h (one 15 min pulse per cycle) is much shorter than in its sibling released from $T=25$ h.

The period (T) of any entraining cycle causes an after-effect on pacemaker period even when T is the natural 24 h. When a group of rodents is released from entrainment by the standard LD 12 : 12 cycle, the values of τ observed in the early part of the freerun are typically within a narrow range around 24 h. In the course of time they drift apart to their characteristic individual steady-state values. Figure 10 demonstrates for *Peromyscus leucopus* that following entrainment to LD 12 : 12, τ is initially closer to 24 h than it is after several months in DD: entrainment to $T=24$ had brought τ away from its otherwise “most probable” value, τ_p . The effect is less clear in *Peromyscus maniculatus* (Fig. 17), but here, too, the range of τ -values increases with time after release from entrainment.

Remarkably clear after-effects of entrainment develop when T is 24 h and a single brief (15 min) pulse is used in each cycle. These after-effects of $T=24$ h are discussed (as the “bouncing” phenomenon) in a later paper in this series devoted to entrainment (Pittendrigh and Daan, 1976a).

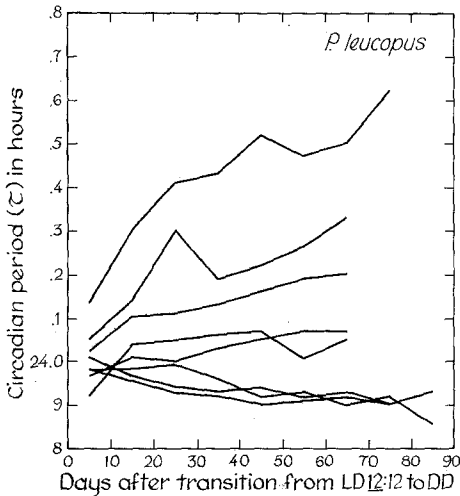


Fig. 10. Changes of τ with time in the course of long DD-freeruns in the white-footed deermouse: τ -values were obtained in 10-day intervals. Each line represents one individual. The animals were 23–27 months old at the start of the freerun

f) After-Effects of Constant Light (LL)

It has long been known that τ of nocturnal rodents placed in constant light is longer than it is in constant darkness (Johnson, 1939; Aschoff, 1951, 1960, 1964). Figure 11 (lower panel) shows that in *Peromyscus leucopus* constant light has a major, consistent after-effect on τ : in DD the pacemaker runs for up to 30 cycles with τ virtually unchanged from its greatly increased value generated by the constant light. It is a significant fact, to which we return later, that hamsters show no such consistent after-effect of LL.

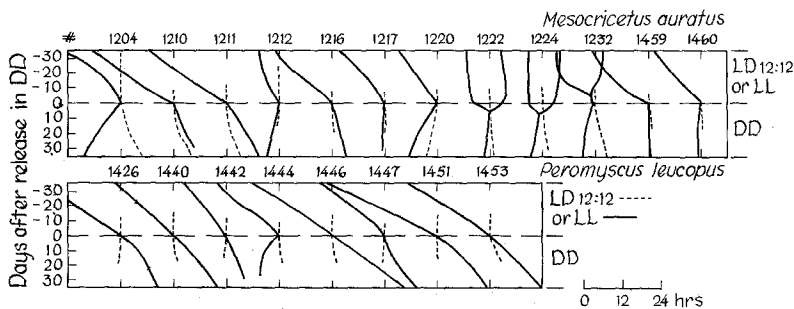


Fig. 11. After-effects on τ in constant darkness (DD) by previous exposure to constant light (LL, 100–200 Lux) in 12 hamsters (*M. auratus*) and 8 white-footed deermice (*P. leucopus*). Heavy lines are eye-fitted to the onsets of activity from 30 days before till 30 days after the transition from LL to DD. In three hamsters (# 1222, 1224, 1232) double lines indicate that the rhythm was split in two components during LL. Dashed lines are for the same animals before and after a transition from LD 12:12 to DD: The lines are horizontally adjusted such that activity onset at the LD 12:12 \rightarrow DD transition and at the LL \rightarrow DD transition are the same. In 3 out of 12 hamsters (# 1210, 1211, 1216) and in 7 out of 8 deermice (all except # 1444) τ was longer after LL than after LD 12:12

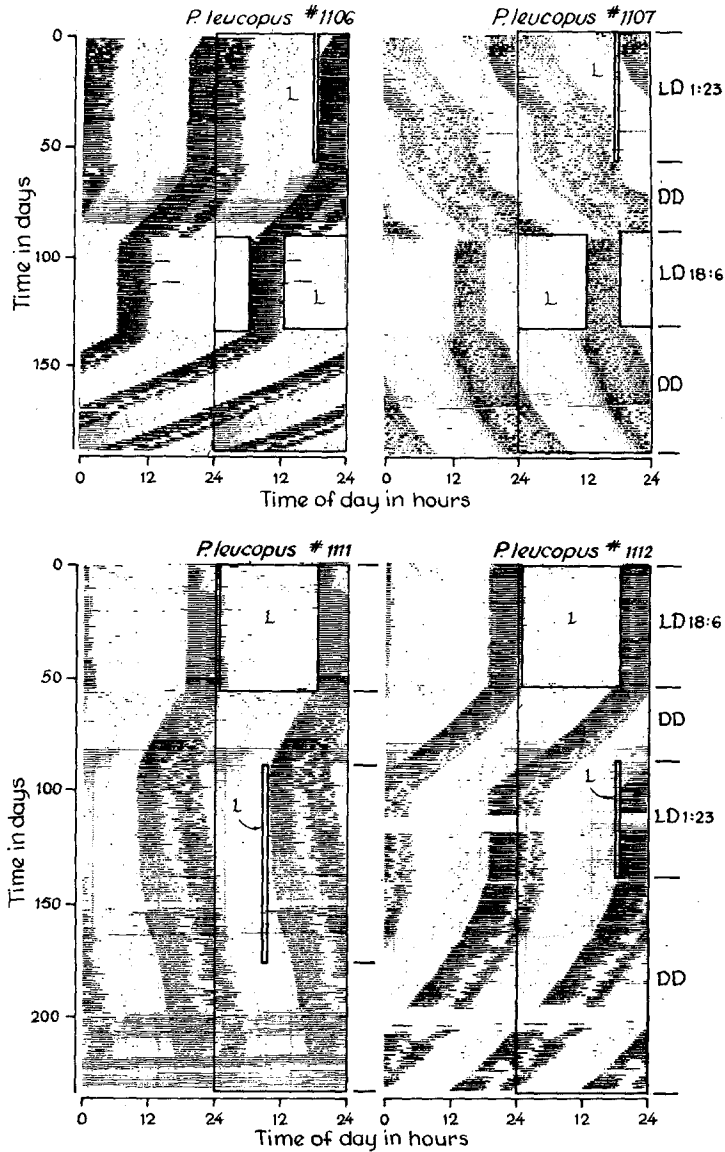


Fig. 12. After-effects of photoperiod in four white-footed mice. The free-running circadian rhythm in constant darkness (DD) was assayed after entrainment to a light/dark cycle with 1 and with 18 h of light. Notice the shorter τ after the long photoperiod. A fifth animal had shorter τ after the short photoperiod (see text). Notice further: the gradual "decompression" of the activity time after the long photoperiod in *P. leucopus* #1107; and the "bounce" (temporary loss of entrainment) in *P. leucopus* #1111

g) After-Effects of Photoperiod

Since after-effects are generated not only by constant light but by the period ($T=24$) of the normal light cycle the pacemaker experiences, it was expected that

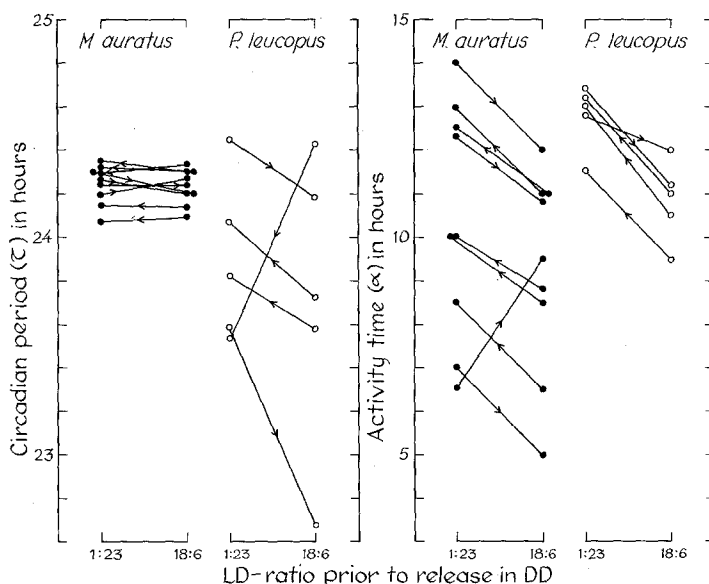


Fig. 13. After-effects of different photoperiods on τ and α in hamsters and white-footed deermice. Results obtained in an experimental protocol as indicated in Figure 12. Lines connect two observations in the same individual. Arrows indicate their sequence

the duration of the photoperiod in such a cycle would also exert an after-effect. In view of the LL after-effects, we anticipated that long photoperiods would lengthen τ . The presumption that the effect on the pacemaker of photoperiod duration is predictable from the effect of constant illumination, is well-imbedded in the circadian literature (e.g. Aschoff and Wever, 1962; Aschoff, 1964) and plays a significant role in the model of entrainment developed by Wever (1964, 1965).

Figures 12 through 15 summarize our observations. The after-effect of photoperiod was first detected in *Peromyscus leucopus*, where the after-effect of LL is so clear. Figures 12 and 13 show that in 4 out of 5 animals studied τ was shorter in freeruns following a long photoperiod than in those following a short photoperiod. The photographed activity record of the exceptional animal was too poor to merit inclusion in Figure 12. It is, however, noted that its exceptional behavior is in fact due to another after-effect phenomenon ("bouncing") associated with very brief light pulses which we discuss in some detail in a later paper (Pittendrigh and Daan 1976a).

In comparable experiments with 9 hamsters we again (as after LL) found no clear after-effect of photoperiod on τ ; however, photoperiod does have impact on α in *Mesocricetus* as it also does in *P. leucopus* (Fig. 13).

In recent experiments with *Mus musculus* that pursue the *Peromyscus* behavior, we have (i) avoided the very short photoperiods that generate "bounces", (ii) concentrated on variation of photoperiod within the natural range, and (iii) sought to distinguish between the parametric and nonparametric effects of the long light signal. Sample raw data of these experiments are shown in Figure 14. The upper panel of Figure 15 gives the data for 10 mice whose τ was measured following

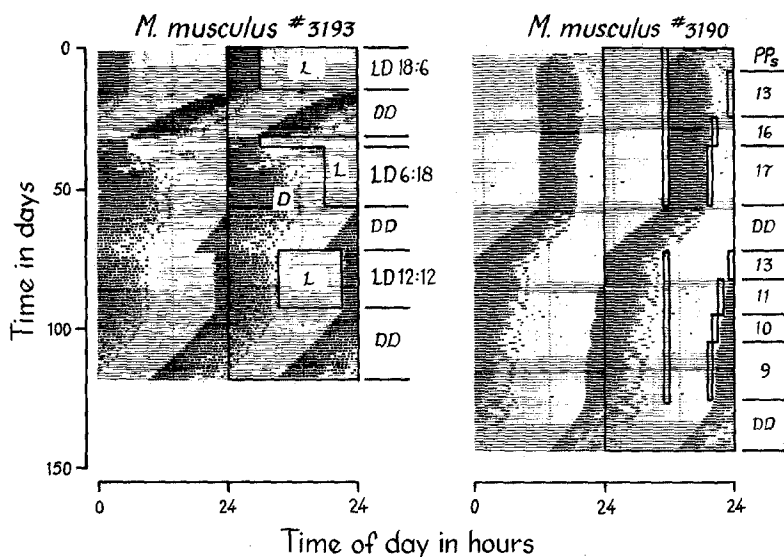


Fig. 14. After-effects of complete photoperiods (#3193) and of skeleton photoperiods (#3190) in *Mus musculus* (C 57). #3193 was entrained successively by LD 18:6, LD 6:18, and LD 12:12; in the intermediate DD-freeruns, τ was shortest after the longest photoperiod (LD 18:6). #3190 was entrained twice to LD 1:7:1:15. τ was shorter in the first DD run, when its activity had been in the 7-h dark interval (PPs 17 including the light pulses) than in the second DD-run, when its activity had been in the 15 h dark interval (PPs 9)

three different photoperiods: LD 6:18; LD 12:12; and LD 18:6. There is a steady decline in τ of the freerunning pacemaker as the length of the prior photoperiod is increased (Table 5). The lower panel of Figure 15 is derived from experiments exemplified by the raw data given in the right panel of Figure 14. Here we varied the length of a so-called *skeleton* photoperiod (cf. Pittendrigh and Minis, 1964; Ottesen et al., in preparation) which consists of two brief (1 h) pulses per cycle. The duration (PPs) of the skeleton photoperiod is the interval between the beginning of one pulse and the end of the second. It will be noted that in choosing PPs 7:17 and 15:9 we are using precisely the same light cycle. The only distinction between them concerns which of the two pulses in each 24 h light cycle is taken by the pacemaker to function as the photoperiod's end (Fig. 14). In such a protocol, the pacemakers will usually be entrained with the active period in the longer of the two dark intervals. To get every animal in one of two runs entrained to this cycle with its active period in the short interval, we had to move one pulse gradually forward, as shown in Figure 14 (right panel). All 13 animals remained for 3 weeks entrained to both skeleton photoperiods before release in DD. There is a clearly different after-effect of the identical skeleton photoperiods: prior entrainment shortens τ , as immediately expressed in the subsequent freerun, when the mice have their activity in the short interval, but lengthens τ , when their subjective night coincides with the long interval (Table 5).

This experiment clarifies the apparent conflict in the different signs of LL and photoperiod after-effects. By definition we must conclude that the lengthening of τ in constant illumination is due to a parametric effect on the pacemaker: no

Fig. 15. Summary of the after-effects of complete photoperiods (top panel) and skeleton photoperiods (bottom panel) in *Mus musculus* C57. Lines connect three or two individual τ values, measured in protocols as in Figure 14. Circles are the group averages for each prior condition

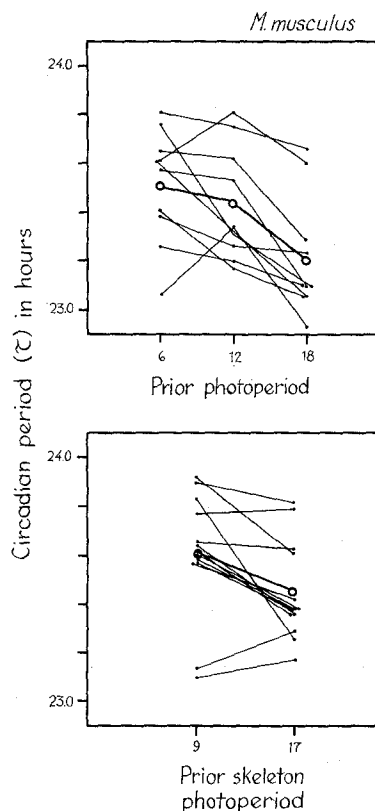


Table 5. After-effects of photoperiod in *Mus musculus* C57. $\Delta\tau$ is the average difference between individual measured after LD 6:18 and LD 18:6, or after PPs 9 and PPs 17

Complete Photoperiods			Skeleton Photoperiods		
	$\bar{\tau} \pm \text{s.e.}$	(n)		$\bar{\tau} \pm \text{s.e.}$	(n)
LD 6:18	23.51 ± 0.07	(10)	PPs 9	23.61 ± 0.07	(13)
LD 12:12	23.44 ± 0.08	(9)	PPs 17	23.45 ± 0.05	(13)
LD 18:6	23.21 ± 0.08	(10)			
$\Delta\tau$	0.30 ± 0.07	(10)	$\Delta\tau$	0.16 ± 0.05	(13)
h			h		

change in external conditions occurs throughout its cycle. The after-effect of photoperiod is surprising only if we assume that the parametric action of a long light pulse (photoperiod) is its dominant effect. In *Drosophila pseudoobscura* the characteristically different effect of each photoperiod on the circadian pacemaker can be accounted for by the interaction of the two non-parametric effects due to the transitions at the beginning and end of each photoperiod (Pittendrigh and Minis, 1964). Figure 15 establishes that the after-effect of photoperiod on our rodent pacemakers is similarly attributable to the interaction of non-parametric effects at the beginning and end of the photoperiod.

h) Intra-individual Variation: The Effect of Age

We have recently reported briefly that τ decreases systematically with age in three of our rodents (*M. auratus*, *P. maniculatus*, *P. leucopus*) (Pittendrigh and Daan, 1974). 10 *P. maniculatus* and 8 hamsters were released from prolonged (ca. 3 months) entrainment by an LD 12:12 cycle in constant darkness twice in their lifetime, and left freerunning for ca. 3 months. The differences are most pronounced in *P. maniculatus*. Figure 16 shows that except for one deermouse (#1505) all animals had shorter τ the second time than the first time. τ -values tabulated for these animals per 10-day intervals (Fig. 17) show that there is small interindividual variation (range ~ 1 h) immediately after release from LD 12:12. This range expands to almost 2 h in 80 cycles, but around a smaller average in the older ani-

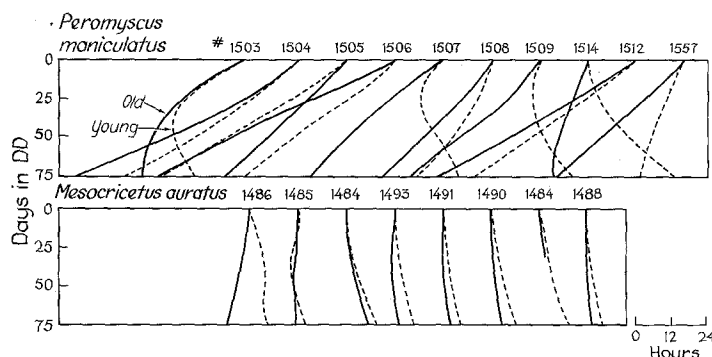


Fig. 16. The effect of age on τ in two species of rodents. 10 *P. maniculatus* and 8 *M. auratus* were transferred from prolonged (>3 months) LD 12:12 entrainment to DD twice in their lifetime. Lines are eye-fitted through the onsets of activity in the first 75 days of DD. The first freerun (*P. maniculatus* 4 months old at day 0, *M. auratus* 5 months) is indicated by dashed lines, the second (*P. maniculatus* 13 months, *M. auratus* 15 months) by solid lines

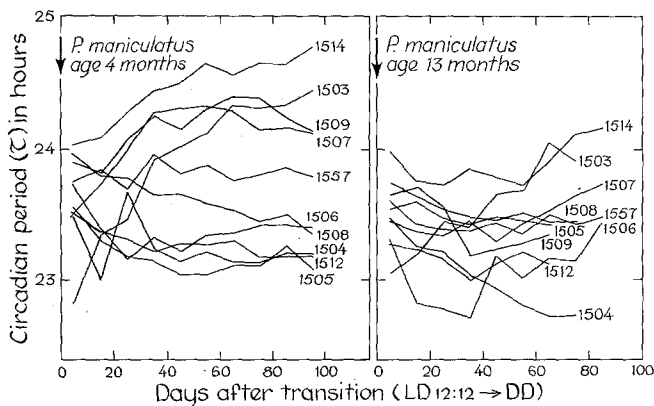


Fig. 17. The time course of τ in long DD-freeruns following prolonged LD 12:12 entrainment at two different ages in deermice. Lines connect τ -values for 10-day intervals in individuals. Animal numbers are indicated. τ -values for day 20–30 are those reported by Pittendrigh and Daan (1974, Fig. 2)

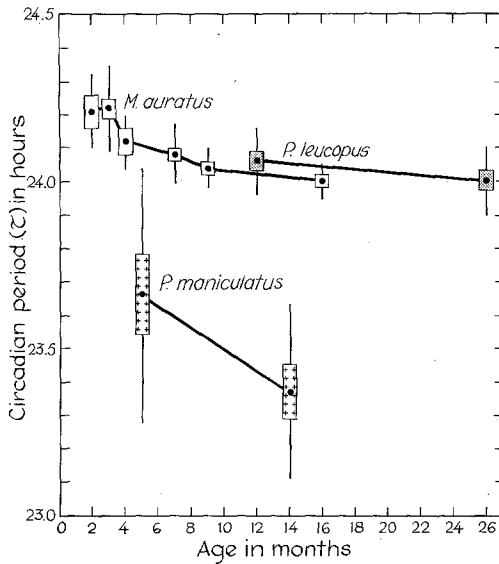


Fig. 18. Summary statistics on τ_{DD} as a function of age in three species of rodents. Means (●), standard deviations (lines) and standard errors (rectangles) are shown for data published extensively by Pittendrigh and Daan (1974). Individual τ -estimates were made for day 20–30 (in *P. leucopus* day 0–10) of a DD-freerun following prolonged entrainment to LD 12:12

mals. Here again we observe the decay of after-effect due to the prior entrainment to LD 12:12 superimposed on the interindividual differences in $\bar{\tau}$. It will be noted (Fig. 17) that individual differences in pacemaker frequency tend to be maintained even as $\bar{\tau}$ changes with age: individuals with above-average τ as young animals (#1514, 1503) are again above-average as old animals. Individuals with below-average τ as young animals (#1504, 1512) are again below average as old animals.

A shorter τ at older age was also observed in 8 out of 8 *P. leucopus*, although here the first DD-freerun lasted only 15 days, and the differences among them were small due to the after-effect of the prior LD 12:12 entrainment.

Figure 18 summarizes the statistics on these experiments, including 51 τ -estimates from hamsters of known age. The hamster data seem to exclude the possibility, open for the other species, that the effect we attribute to age may be traceable to other origins, such as annual fluctuation in τ . Thus while the data for “young” and “old” individuals in Figures 16 and 17 were not collected at precisely the same season, those for the hamster in Figure 18 derive from freeruns scattered throughout the year and $\bar{\tau}$ shows a clear downward trend with age.

The facts summarized by Figure 19 also argue against any circannual or other alternative interpretation. The figure records $\bar{\tau}$ and its standard error derived from two batches (each of 6 male animals) of *Mus musculus* (strain C57BL/6J). One batch was 6 months old, the other 26 months old at the beginning of the experiment. They were obtained from a special colony (Charles Rivers, Boston, Mass.) maintained for gerontological research. They are highly inbred and are maintained under rigorously standardized conditions and diet. We subjected both batches

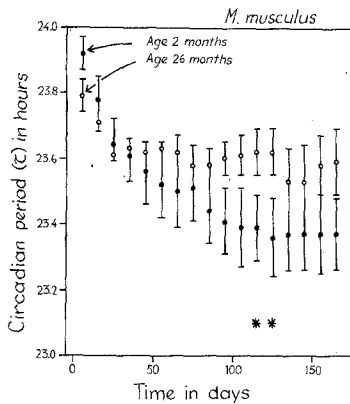


Fig. 19. Change of freerunning period (τ) with time in DD in old (\bullet) and young (\circ) mice. Ages indicated are those on day 0, at release from LD 12:12. Vertical lines are ± 1 standard error of the group means. Asterisks indicate where $p < 0.1$ (t -test)

to LD 12:12 for two weeks before releasing them into DD. As with all our other animals, τ was initially much closer to 24 h than is characteristic of the species after a prolonged freerun in DD. The after-effect of being entrained to $T = 24$ h gradually decayed, and as it did it became increasingly clear that τ in the older animals was shorter than in the younger. The group differences never became statistically significant; the sample size was evidently too small, given the range of interindividual variation. The sign of the difference is the same as in the other three species.

The activity time (α) can be measured with much less accuracy than τ . Figure 20 suggests that, if there is any trend in α with age, it is toward shorter activity time

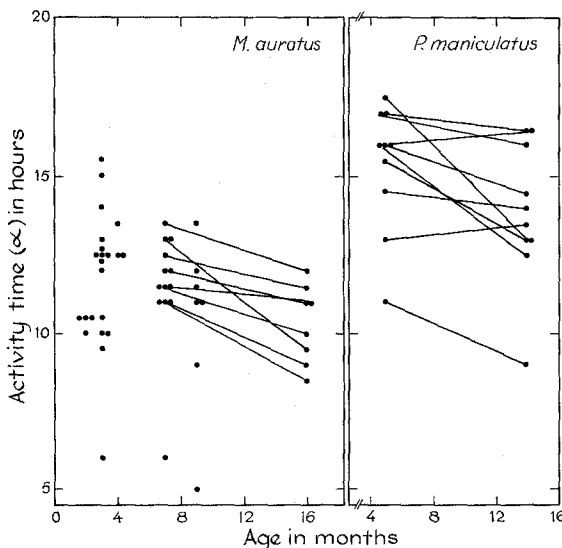


Fig. 20. Activity time in DD as a function of age in two species of rodents. Activity time was estimated on days 20–30 of a DD-freerun following prolonged entrainment to LD 12:12. Lines connect observations for individuals tested twice in their lifetime

in older animals, in both hamsters and deermice. This effect is most likely related to the well-known general decline in total running-wheel activity with age (e.g. Richter, 1922; Jones et al., 1953; Aschoff, 1962). It is another case where the negative correlation between τ and α embodied in Aschoff's Rule turns out not to be general (cf. Lohmann, 1967).

IV. Discussion

Much of the discussion which these properties of circadian pacemakers invite concerns their functional significance; they relate to the role of circadian oscillators as clocks. That discussion, however can only be undertaken after presentation of other empirical regularities concerning inter- and intraspecific variation in the oscillator's phase-response-curve, and its dependence on τ (Daan and Pittendrigh, 1976a). Other aspects of our data are, however, of more general interest.

a) Age

Until recently there was no evidence that any property of a circadian pacemaker, as distinct from the pattern and amplitude of the rhythm it drives, was affected by aging. The fact that in all 3 species we initially examined (Pittendrigh and Daan, 1974) τ shortened as the animal aged suggested the effect may be widespread: and that likelihood is now increased by finding it in *Mus musculus*.

The effect has only been looked for in male rodents. It is known that testosterone levels have a slight effect on pacemaker frequency (Daan et al., 1975) and testosterone levels are known to change with age (e.g. Guyton, 1971, Fig. 80-8). However, the aging effect cannot be attributed simply to testosterone itself. Its effect in mature males is to shorten τ ; or, more precisely, τ lengthens following castration, and gradually becomes shorter when testosterone-loaded capsules are implanted (Daan et al., 1975). In hamsters the greatest decline in circadian period attributable to age is in young males; it is tempting to attribute that pubertal change to the rising testosterone level, but the continued steady shortening of τ as the animal ages cannot be attributed to the same cause since testosterone then systematically decreases rather than increases. Nevertheless the proximate, concrete, causes of the age effect may well prove to be endocrinological and that prospect invites further work. So, too, does the possibility raised elsewhere (Pittendrigh and Daan, 1974) that unless all circadian pacemakers in a metazoan change frequency at the same rate as they age, there will be a steady deterioration of "normal" circadian organization that may itself contribute to the more general physiological deterioration which we describe as aging.

b) History-Dependence of Pacemaker State

The precision of circadian activity rhythms in vertebrates has long been known; it makes them especially useful for much experimental work; and, in general, is

recognized as "remarkable". But the pacemaker driving them, when responsible for only half the rhythm's variance, as our analysis suggests, is even more remarkably precise. The rhythm's precision is detectable even in relatively short-term recordings of a week or so, and perhaps for that reason alone it has seemed unnecessary to explore the behaviour of the system freerunning for longer durations. Only Eskin (1971) and Kramm (1971) have previously recorded circadian rhythms in individual animals long enough to detect the kind of lability reflected in the "after-effects" we have described.

The facts reported here on the history-dependence of τ leave no doubt about the importance and regularity of the after-effect phenomenon which has received little attention in the fifteen years since it was first described. The act of entrainment to $T=24$ h, the natural case, has an impact on τ : in the entrained steady-state the "real" value of τ is closer to T than the τ to which the system eventually drifts in prolonged freeruns in darkness. Moreover change of photoperiod within the natural range affects τ .

There is surely functional significance in this response of the pacemaker to the light-cycle that drives it. It is intuitively plausible that the oscillator should adapt its own period (τ) to that (T) of the external world: the phase shifts ($\Delta\phi$) necessary for entrainment are minimized when τ is close to T . But there are problems inherent in this intuitive judgement that can only be adequately pursued later (Pittendrigh and Daan, 1976a). Why, if there is adaptive merit in having τ approach T , has selection set $\bar{\tau}$ in some species far from T in the first place; why a circadian period at all? And why if the adaptive goal is only to have τ approach T , under experience, is the effect of photoperiod tolerated? The effect of 24 h days with short photoperiods in winter evidently is to bring the very short $\bar{\tau}$ of *P. maniculatus* closer to 24 h; but that trend is reversed in midsummer when the after-effect of the long photoperiod is to shorten τ again. While there may well be some validity to the proposition that this particular pacemaker lability is to minimize the "trauma" of daily phase-shifts there is clearly more to it. Indeed, we later conclude that, at present, it is only the after-effect of photoperiod for which functional utility can be found; it is not just tolerated. It is the after-effect of T which may lack functional significance and itself be merely tolerated as an inescapable consequence of that pacemaker property which facilitates lability with respect to photoperiod.

It may be tempting to describe the after-effects on τ —indeed any history-dependent phenomenon—as a case of "learning". We see no utility in that description and raise the point only because the learning approach has muddled issues in this field before, not only in its earlier history (e.g. Semon, 1912) but even in the modern era. Thorpe's discussion following Pittendrigh's (1958) paper and his later treatment of circadian rhythms as being "imprinted" on organisms (Thorpe, 1966, p. 193) indicate the temptation dies hard. The structure of circadian pacemakers, including provision for some lability is completely encoded in DNA as experimentally induced mutations in τ now fully attest (Konopka and Benzer, 1971). What the phenomena of after-effects inform us of is that while the genetically specified system may well have a most-probable state, characterized by its frequency, for any set of prevailing conditions, the realized state one observes at any time is strongly history-dependent; and progress to the most-probable state may be slow if prior conditions established a very different state.

The idea of a most probable state (with τ_p) for the pacemaker in a specified environment (e.g. DD) seems to us useful; it is the state to which the system is drifting, governing the characteristic time course of the freerun in which τ is steadily changing (cf. Figs. 7 and 8). But on the other hand τ_p is certainly not easily measured: when in a prolonged freerun does the observed change in $\bar{\tau}$ stop reflecting an approach to τ_p as distinct from a change in τ_p due to age? At present our best estimate of τ_p for a given environment and a given species such as constant darkness is $\bar{\tau}$, the average of all τ estimates in that environment following a diversity of pre-treatments.

c) On the Design of Circadian Experiments

Our introduction noted that had we known their outcome some of our experiments would have been structured differently. This is a commonplace in empirical work but the usual remedy of redoing an experiment "properly" is not undertaken lightly when the times involved are months or years. It is the substantial variation of $\bar{\tau}$ between individuals within a species, and its history-dependence within the individual, that bears most significantly on the adequacy of experimental design in this field. In choosing, for instance, *Mus musculus* as an object one clearly cannot assume that τ of the untreated pacemaker is known, even from hundreds of previous experiments. Yet such an assumption is not uncommonly made in the literature and underlies shortcomings in one of our subsequent papers on pacemaker entrainment (Pittendrigh and Daan, 1976a): we did not always measure τ in an individual hamster or deermouse before assaying its entrainment responses, and residual discrepancies between observation and a model's predictions cannot therefore be fully evaluated.

The severity of the constraints we confront is illustrated by the facts in Table 6. The freerunning period (in DD) of young males of two highly inbred strains of *M. musculus*, obtained from a reliable dealer, was measured in December 1973. There was a major and highly significant ($p < 0.001$) difference between the $\bar{\tau}$'s of DBA and C57 BL/6S. This difference was thought to provide a reliable tool to explore the dependence on intraspecific τ variation of other pacemaker properties (tightness of homeostatic control, response to light, shape of phase-response-curve). But four months later as those experiments were to begin, the difference was totally undetectable in new batches of the same strains obtained from the same dealer: it is not merely loss of statistical significance; $\bar{\tau}$ in the two strains is essentially identical.

Table 6. Variations in τ in two samples of two strains of *Mus musculus*, DBA and C57. Average $\bar{\tau} (\pm 1$ standard error) in hours; sample size in parentheses. p -values for the significance of sample means based on two-tailed t -test

Sample	Date	Strain: DBA	Strain: C-57	p
1	28 Oct. 1972	23.09 ± 0.09 (9)	23.46 ± 0.08 (9)	< 0.001
2	19 March 1973	23.49 ± 0.04 (17)	23.47 ± 0.05 (17)	> 0.1
	p	< 0.001	> 0.1	

When an experiment on circadian pacemakers employs separate concurrent "controls" and "experimentals", maximization of sensitivity obviously demands that all known sources of extraneous variation be removed. The most obvious of these include sex, strain and age. But there are others, likely to be ignored, that derive from the history-dependence of the individual pacemaker's state. Every detail of prior exposure to light (or other natural entraining agent) is relevant, including the period (T) of the light cycle and its photoperiod as well as the duration of the entrained steady-state. It is often convenient to set different animals to different phases relative to local time so that a specified treatment can be given to different animals at different circadian times within the working day. But even a week of entrainment to some new phase may be insufficient to assure that the different animals are free of after-effects known to be generated by the sign (advance, delay) of the transients leading to the new steady-state. Three weeks of LD 12:12 had still not removed the after-effects on $\bar{\tau}$ of the transients leading to entrainment in the hamsters recorded in Figure 6.

The extents and sources of τ -variation we have encountered prompt another comment on experimental design. Inter-individual variation not only in τ itself but its own lability is such that some effects are unlikely to be detected at all unless the whole approach of concurrent separate "controls" is abandoned and the analysis based entirely on intra-individual comparisons. This point is well made by the data reported here on the effect of age in *Mus musculus*. The change in τ attributable to age is smaller, in all species, than the difference in $\bar{\tau}$ between some individuals in that species. Having found the effect in records from aging individuals in the other three species, it was then sought in *Mus musculus* for which we had no comparable data. The availability of highly inbred C57BL/6J of known age, reared under meticulously standardized conditions for gerontological research, made it possible to pursue the question experimentally without waiting 25 months as intraindividual analyses progressed. A positive intergroup difference was found (Fig. 19) in the expected direction after several months when the after-effect of prior entrainment to LD 12:12 had decayed. But the effect was so imbedded in intraindividual variation that our confidence in its reality would be low ($p < 0.1$) were it not for intra-individual data from the other species.

d) Species Diversity in Pacemaker Lability

Later papers in this series address the *similarities* that appear to characterize circadian pacemakers including the few we have studied. It is appropriate here to stress the *diversity* in pacemaker behavior we have encountered among four closely related organisms including two species in the same genus. *Peromyscus leucopus* and *P. maniculatus* differ not only in $\bar{\tau}$ but in its lability (Fig. 3). Those who feel "it is time to wind up the Biological Clock" (Anonymous, Nature, 1971) have many on their hands. That is not to say our rodent pacemakers are fundamentally different, but that the differences between them are as fundamental an issue as their similarities. Has selection merely tolerated them, or sought them as contributions to different strategies in the mechanism of circadian pacemaker entrainment?

The most striking aspect of interspecies diversity is not so much in the pacemaker's average period ($\bar{\tau}$) itself, although that is clear. It is more remarkable that the species differ so much in both the day-to-day instability of τ and especially in their correlated susceptibility to longer term lability reflected in both after-effects and the dependence of $\bar{\tau}$ on age. Hamsters' pacemakers are uniquely stable, relatively intractable to after-effects and least affected by age. The most remarkable feature, however, is the interdependence of $\bar{\tau}$ and its stability in general. Hamsters with $\bar{\tau}$ indistinguishably close to 24 hours have as noted the most inflexible and stable pacemaker. The further $\bar{\tau}$ gets from 24 h the less precise (day-to-day) and stable it becomes. Aschoff et al. (1971) have presented evidence from birds and man, suggesting that the same principle applies to the day-to-day precision in individuals when τ is modified by varying the external conditions.

The bearing of all these facts on entrainment strategies and the problems they raise in developing a minimally adequate model of the pacemaker are topics to which we return later (Pittendrigh and Daan, 1976a, b). But they raise another, distinct question. Do they not imply that the general homeostasis of frequency (Pittendrigh and Caldarola, 1973) is tightest in *Mesocricetus* and progressively looser in the other species? If so we should expect the species will differ in the extent to which $\bar{\tau}$ can be changed by any experimental procedure including the standard tools, constant light and D₂O (Daan and Pittendrigh, 1976).

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